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Role of antioxidant enzyme responses and phytochelatins in tolerance strategies of *Alhagi camelorum* Fisch growing on copper mine

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This study was performed to clarify some aspects of tolerance mechanisms against excess copper (Cu) in *Alhagi camelorum* Fisch, a dominant wild type plant growing in a Cu-contaminated zone and its vicinity. Total and available copper was at toxic levels for plants growing on the contaminated soil. There were no visual and conspicuous symptoms of Cu toxicity in this plant species. Most of the excess Cu in soil was transferred to and accumulated in plant leaves in which the storage rate in vacuoles and chloroplasts was 48% and 7% respectively. There was an insignificant decrease in chlorophyll content and a significant increase in tissues phytochelatins and antioxidant enzyme activities in plants collected from the contaminated zone as compared to plants of the same species growing on uncontaminated soil. We also observed significant elevation in oxidative damage biomarkers, malondialdehyde and dityrosine, when the aerial parts of *Alhagi camelorum* were compared with the same parts of the plant collected from an uncontaminated zone. *Alhagi camelorum* elevated its antioxidative enzyme activities, phytochelatins and accumulated the excess of Cu in leaf vacuoles in response to Cu-toxicity as tolerance strategy.

Key words: Copper, metal, toxicity, antioxidant, phytochelatin, tolerance

Abbreviations: CAT – catalase, DTPA – Diethylene triaminepenta acetic acid, EC – electric conductivity, GPX – glutathione peroxidase, GSH – glutathione, HEPPS – (3-[4-(2-Hydroxyethyl)-1-piperazinyl] propanesulfonic acid), MDA – malondialdehyde, MSA – methanesulfonic acid, NBT – nitroblue tetrazolium, PC – phytochelatin, ROS – reactive oxygen species, SOD – superoxide dismutase, TBA – thiobarbituric acid, TFA – trifluoro acetic acid

Introduction

Copper is an essential micronutrient for plants and a component of several enzymes mainly participating in electron flow, catalyzing the redox reactions in mitochondria and chloroplast. In addition, this element is also a cofactor or a part of a prosthetic group of many key enzymes that play important roles in photosynthesis, respiration, CO_2 assimilation, ATP synthesis and nitrogen metabolism (DEMIREVSKA-KEPOVA et al. 2004). The ab-

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sorption of copper from soil by plants and its transport to the above-ground parts depend on the ability of plants to transfer this metal across the soil-root interface and to the total amount of Cu, present in the soil (AGATA and ERNEST 1998, BAKER and PROCTOR 1990). On the other hand, copper is a widespread contaminant originating from different human activities including mining and smelting of copper ores. Mining activities generate a large amount of waste rocks and tailings, which get deposited on the surface. In this condition, a network of sequestration activities and immobilization functions regulate the uptake, distribution and detoxification of excess metal ions in plants (CLEMENS 2001).

An active detoxification mechanism acts by means of glutathione (GSH) and of peptides synthesized at the expense of GSH, the phytochelatins (PCs), (DE VOS et al. 1992). Glutathione (GSH) is a low-molecular weight thiol tripeptide, involved in cellular defense against the toxic action of xenobiotics, oxyradicals as well as of metal cations (MEISTER and ANDERSON 1983). It is able to modify metal toxicity by chelating metal ions in cells; it plays a key role in protecting macromolecules from damage by free radicals by trapping them in an aqueous phase (FREEDMAN et al. 1989).

On the other hand, PCs are polymers of γ-Glu-Cys units with the terminal glycine and capable of binding heavy metals via thiolate coordination. They are induced in response to many heavy metals including copper (MORELLI and SCARANO 2004) when the non-PC based mechanism of detoxification gets exhausted and free metal ions become available to induce PC synthesis (SCHAT et al. 2002).

Exposure to excess Cu is capable of stress induction in which the role of oxidative stress and reactive oxygen species (ROS) production may be involved (STADMAN and OLIVER 1991, WALDERMAR et al. 1994). On the other hand, under Cu toxicity, excess copper is an efficient generator of ROS in Fenton-type reactions, leading to disturbance in metabolic pathways and macromolecule damages (HEGEDUS et al. 2001).

ROS are partially reduced forms of atmospheric oxygen and under normal conditions their production in plant cells is tightly controlled by the scavenging system. ROS can oxidize biomolecules such as DNA, proteins and lipids, creating oxidative injury that results in a reduction of plant growth and development (HERNANDEZ-JIMENEZ et al. 2002, OGAWA and IWABUCHI 2001).

Since the half-lives of ROS are extremely short, their stable end products of oxidative damage to cellular macromolecules can be used for oxidative stress monitoring (ORHANL et al. 2004). Dityrosine, a hallmark of oxidized proteins and malondialdehyde (MDA) a biomarker of lipid peroxidation are closely correlated with oxidative stress level (HALLIWELL and GUTTERIDGE 1998).

To control the level and effects of ROS, cells have developed various antioxidant defenses including antioxidant enzymes and low molecular mass radical scavengers. They regenerate the active form of antioxidants and eliminate or reduce the damage caused by ROS (ALSHER et al. 1997).

Among plants growing on Cu-contaminated soils, Cu-tolerant genotypes evoke antioxidant enzyme induction as a general response to the toxic effects of copper to protect them against metabolic disturbance and cellular damage (VAN ASSCHE and CLIJSTERS 1990).

Superoxide dismutase (SOD), the first major enzyme found in all aerobes, catalyses dismutation of superoxide anion to H_2O_2 and molecular oxygen. Glutathione peroxidase protects the membrane lipids from oxidative damage and detoxifies the organic peroxides;

it can also act on organic hydroperoxides (KANTOL et al. 1998). The intracellular level of H_2O_2 is regulated by a wide range of enzymes, the most important being catalase and peroxidase. Catalase inactivates H_2O_2 to oxygen and water (RUSINA et al. 2004).

In the present study, field surveys have been carried out on *Alhagi camelorum* plant growing in the area of a copper mine in Kerman state. The aim of this study was to investigate the Cu-accumulating ability of this wild type plant growing around the mine. We also evaluated the corresponding status of the antioxidant enzyme activities, the levels of phytochelatins, GHS and oxidative damage products of lipids and proteins to clarify some aspects of this plant toxicity tolerance.

Materials and methods

This study was carried out at Sarcheshmeh, located in Sirjan at Kerman province in Iran (Longitude: 55°, 52', 20'' E, Latitude: 29°, 56', 40'' N). A maximum temperature of +32 °C and an average annual air temperature of 15 °C were recorded. The annual rainfall was about 516 mm and there was no industry nearby. After a geobotanical survey, two zones were considered for plant and soil sampling. Zone 1 was located in center of the Cu-mine area and Zone 2 was approximately 9.7 km south of the waterlogged area of the Cu-mine and the ecological conditions were similar in both zones. The soil of zone 2 was taken as a control because it had never received sources of Cu. Zone 1 was one of the well-known copper mines where the main activity was copper extraction at %1.16 grade of copper. Tailings had been abandoned for 11 years at the time of sampling.

The levels of Cd, Co, Zn and Pb in zone 1 were generally below the USA maximum tolerant concentrations (KABATA-PENDIAS 1995).

The ratio of total Cu in zone 1 to that in zone 2 was about 52-fold. The ratio for available Cu was about 83 fold, showing that the available level was higher than toxicity threshold levels (ICRCL 1987).

Soil and plant sampling

Alhagi camelorum Fisch was endemic and widespread in the study area. At each zone, plant samples were collected at a determined time of a single growing season and according to the actual landform of the copper-mine and the distribution of vegetation before flowering period. Care was taken to collect plant species samples from both zones while they were in growth period. We established three random regions in each study zone. We collected at least 5–8 plants of our species from each area. Fresh tissues including roots, stems and mature leaves of collected plants were considered for three replicate analysis.

Plants were cleaned in abundant deionised fresh water, rinsed with distilled water and identified by an expert botanist. Care was taken to avoid sample contamination with other sources of Cu during sampling, washing, transporting and analysis. Corresponding soil samples were also collected at the location of plant sampling from the rooting zone (maximum sampling depth about 30 cm) and transferred to a polythene bags. Excess air was squeezed out, the bags were sealed, transferred to the laboratory and stored at 4 °C for a maximum of 48 h prior to analysis. These samples were then air-dried and sieved through a 2 mm plastic screen. There were 6 replicates for each soil sample.

Soil analysis

Dried soil samples were digested with HCl + HNO_3 + $HClO_4$ (3:1:1, w.v.) (YUAN 1988). Total Cu and other metals were determined by atomic absorption spectrophotometer (Analyst 100, Perkin Elmer, USA), using an acetylene- air flame. Diethylenetriaminepentaacetic acid (DTPA)-extractable Cu, Cd, Co, Zn and Pb contents of 10 g soil samples (sample: DTPA, 1:2, w.v.) were determined by atomic absorption spectrophotometer (PAGE et al. 1982).

The reagents and standards for AAS were ultra pure. The detection limits in mg kg^{-1} for total and extractable metals in soils were: 0.06 for Cd, 0.15 for Co, 0.17 for Pb, 0.08 for Cu and 0.11 for Zn. This step represents the fraction that is water soluble and most easily available to plants and easily leachable into the ground water (SIEB 1995). Soil nitrate (NO_3^-) was analysed colorimetrically (KEENEY and NELSON 1982). The total nitrogen was determined by the Kjeldhal digestion method (DUCHAUFOUR 1970). The pH and electrolytic conductivity (EC) were determined in a water:soil extract 1:1 using a Beckman pH-meter and a conductivity meter model HI8633, Hanna Instruments Co., respectively.

Plant biomass and Cu content analysis

The washed plant samples were separated into roots and shoots, and dried in an oven at 60 °C for 48 h; then the biomass presented as dry weight was measured. For elemental analysis, the dried plant tissues were ashed in a muffle furnace at 550 °C for 24 h. The ash was digested with a mixture of HNO_3 and $HClO_4$ (5:3 wet weight), heated in an oven. After cooling, the extracts were diluted and made up to 25 mL with 1 M HNO_3. Copper concentration of the extract was determined by atomic absorption spectrophotometer.

Chlorophyll determination

Fresh and mature leaves (0.5 g) were extracted with 10 mL 80% acetone as described by ALAN (1994). The absorbance of extract was measured at 663 and 645 nm in the UV-Vis light spectrophotometer (model UV-9100). The chlorophyll content was calculated using the equation as follows:

$$C_T = 20.2\ A_{645} + 8.02\ A_{663}$$

Chloroplast isolation

Fresh and mature leaves (5 g) were homogenized for 15 s with a homogenizer in 50 mL ice-cold grinding medium containing: 0.33 M sorbitol, 1mM EDTA, 0.1% BSA, 2 mM sodium ascorbate and 50 mM K_2HPO_4, pH 7.5. The homogenate was filtrated through Miracloth and centrifuged for 1 min at 1000 g at 4 °C to remove whole cells and cell debris. The intact chloroplasts were pelleted through centrifugation at 4500 g for 30 s and were gently resuspended in the same buffer without BSA and centrifuged again at the same conditions. This washing procedure was repeated twice and pelleted chloroplasts were isolated (RUSINA et al. 2004).

Vacuole isolation

Leaves were floated on an enzyme solution containing 1 mM $CaCl_2$, 500 mM sorbitol, 0.05% (w.v.) polyvinyl pyrrolidone, 15 mM MES/Tris pH 5.5, 0.2% (w.v.) bovine serum

albumin, 1% (w.v.) cellulose, 0.5% (w.v.) Macerozym, 0.01% (w.v.) pectolyase, and agitated for 30 min. Vacuoles were released into the recording chamber by hyposmotic shock treatment of protoplasts in 100 mM KCl, 5 mM $MgCl_2$, 2mM EGTA, 1 mM dithiotheritol (DTT) and 5 mM Tris/MES, pH 7.5, adjusted to π = 300 mOsm with D-sorbitol. After setting of the vacuoles, the hypotonic solution was carefully replaced by standard bath solution (SCHOLZ-STARKE et al. 2004). Measurement of dityrosine: 1.2 grams of fresh tissue material were homogenized with 5 mL of ice-cold 50 mM HEPES-KOH, pH 7.2, containing 10 mM EDTA, 2 mM PMSF, 0.1 mM p-chloromercuribenzoic acid, 0.1 mM DL-norleucine and 100 mg polyclar AT. The plant tissue homogenate was centrifuged at 5000 g for 60 min to remove debris. Purification of *o*,*o'*-dityrosine in the clear tissue homogenized supernatant fluid was accomplished by preparative HPLC.

o,*o'*-Dityrosine was recovered by gradient elution from the C-18 column (Econosil C18, 250 mm × 10 mm) (ORHANL et al. 2004). The composition of eluent varied linearly from acetonitrile–water–TFA (1:99:0.02) to acetonitrile–water– TFA (20:80:0.02) over 25 min. The gradient was started 5 min after the injection. A flow rate of 4 ml/min was used. *o*,*o'*-Dityrosine was analyzed by reversed-phase HPLC with simultaneous UV-detection (280 nm) and fluorescence-detection (ex. 280 nm, em. 410 nm). A phenomenex inertsil ODS 2 (150 mm × 4.6 mm, 5 μm) HPLC column (Bester, Amsterdam, the Netherlands) equipped with a guard column was used for these analyses. A gradient was formed from 10 mM ammonium acetate, adjusted to pH 4.5 with acetic acid, and methanol, starting with 1% methanol and increasing to 10% over 30 min. The flow rate was 0.8 mL min^{-1}. A standard dityrosine sample was prepared according to AMADO et al. (1984). Dityrosine was quantified by assuming that its generation from the reaction of tyrosine with horseradish peroxidase in the presence of H_2O_2 was quantitative (using the extinction coefficient e_{315} = 4.5 mM^{-1} cm^{-1} at pH 7.5).

Malondialdehyde analysis

Proteins of tissue homogenate were precipitated with 40% trichloracetic acid (TCA), w.v. The MDA assay was based on the condensation of one molecule malondialdehyde with two molecules of thiobarbituric acid (TBA) in the presence of reduced reagent volumes to increase sensitivity, generating a chromogen with UV absorbance. The TBA + MDA complex was analyzed by HPLC (BIRD et al. 1983). HPLC system consisted of a Hewlett + Packard 1050 gradient pump (Avondale, PA) equipped with an automatic injector, a 1050 diode-array absorption detector and a personal computer using Chem Station Software from Hewlett + Packard. Aliquots of the TBA + MDA samples were injected on a 5 mm Supelcosil LC-18 reversed phase column (30 × 4.6 mm). The mobile phase consisted of 15% methanol in double-distilled water degassed by filtering through a 0.5 μm filter (Millipore, Bedford, MA).

The flow rate was 2 mL min^{-1}. MDA + TBA standards were prepared using tetraethoxypropane. The absorption spectra of standards and samples were identical, with a characteristic peak at 540 nm. Measurements were expressed in terms of malondialdehyde (MDA) normalized to the sample protein content. Protein content was determined by the method of Bradford, with standard curves prepared using BSA (BRADFORD 1976).

Extraction and determination of PCs and GSH were performed according to SNELLER et al. (2000). Frozen plant tissues were homogenized with a pestle and mortar with quartz sand in 2 mL of 6.3 mM diethylene triaminepenta acetic (DTPA) with 0.1% trifluoro acetic

acid (TFA) at 4 °C. The homogenate was centrifuged at 14,000 g at 4 °C for 12 min. The clear supernatants were collected for the assay by HPLC using pre-column derivatization with a fluorescent probe, monobromobiane (mBrB). 250 μL of supernatant was mixed with 450 μL of 200 mM HEPPS (3-[4-(2-Hydroxyethyl)-1-piperazinyl] propane sulfonic acid) at pH 8.2, with 6.3 mM DTPA, and 10 μL of 25 mM (mBrB). Derivatization was carried out in the dark at 45 °C for 30 min. The reaction was terminated by the addition of 300 μL of 1 M methane sulfonic acid (MSA). The samples were stored in the dark at 4 °C until HPLC analysis. Blank samples were used to identify the reagent peaks. The bimane derivatives were separated using a binary gradient of mobile phase A (0.1% TFA) and B (100% acetonitrile) at room temperature (22 ± 2 °C). Fluorescence was detected at 380 nm excitation and 470 nm emission wavelengths. The flow rate was 0.5 mL /min. Fifty μL of the derivatives sample was run in a linear gradient from 12% to 25% B for 15 min, then 25% to 35% B for 14 min and next 35% to 50% B for 21 min. Before a new sample was injected, the column was cleaned (5 min, 100% B) and equilibrated (10 min, 12% B) and post-time was 5 min. Total analysis time was 70 min. Analytical data were integrated by using the HP ChemStation. Retention times of PCs and GSH in biological samples were checked with PCs and GSH standards, respectively. Individual PC subtypes were quantified by using the relationship peak vs. concentrations of GSH standard solutions. Corrections for differential derivatization efficiencies were made according to the method stated by SNELLER et al. (2000).

Preparation of enzyme extracts

Whole tissue (leaves, stems and/or roots) were homogenized (1:5 w.v.) separately in an ice cold mortar using 50 mM sodium phosphate buffer, pH 7.0, containing 1 M NaCl, 1% polyvinylpyrrolidone and 1 mM EDTA. After centrifugation (20,000 g, 15 min), the supernatant (crude extract of leaves) was used to determine enzyme activities, which were measured at 25 °C.

Enzyme assays

Catalase (EC 1.11.1.6) activity was determined by following the consumption of H_2O_2 (extinction co-efficient 0.0394 mM mL^{-1}) at 240 nm for 30s (AEBY 1984). The assay mixture contained 100 mM potassium phosphate buffer (pH 7.0), 15 mM H_2O_2 and 50 μL leaf extract in a 3 mL volume. Unit was defined as μmol H_2O_2 decomposed per 1 min. To detect glutathione peroxidase [EC 1.11.1.9 (GSH-Px)] activity, the method of Hopkins and Tudhope, with t -butyl hydroperoxide as a substrate was used (HOPKINS and TUDHOPE 1973). The reaction mixture comprised 50 mM potassium phosphate buffer, pH 7.0, 2 mM EDTA, 0.28 mM NADPH, 0.13 mM GSH, 0.16 U GR, 0.073 mM t-butyl hydroperoxide and enzyme extract (50 mg protein). One unit of GSH-Px activity was defined as the amount of enzyme that catalyzed the oxidation of NADPH [mmol min^{-1} mg^{-1} protein]. SOD activity was determined according to MINAMI and YOSHIKAWA (1979), with 50 mM Tris-Ca-codylic sodium salt buffer, pH 8.2, containing 0.1 mM EDTA. The reaction mixture was composed of 1.42% Triton X-100, 0.055 mM nitroblue tetrazolium (NBT), 16 mM pyrogallol and enzyme extract (50 mg protein). The principle of this reaction is based on the measurement of the concentration of the reduced form of NBT determined at 540 nm. The 50% inhibition was established according to MCCORD and FRIDOVICH (1969). This

inhibition was defined as the quantity of enzyme required to inhibit the reduction of NBT by 50% per 1 min.

Statistical analysis

All statistical analysis was carried out by using the procedure available in the SPSS v.10 (SPSS INC., Chicago, IL) statistical package. Each experiment was run on each sample at least in three replicates from all samples collected from all parts for each zone. Student^{ž}s t-test was applied to determine the significance of results between different samples.

Results

Evaluations of pH and EC level revealed that the water extracts of the soils in both zones were mild acidic and there was no problem with salinity; however the soil samples associated with plants in zone 1 had a slightly lower pH than zone 2. To better characterize nitrogen species levels, we evaluated total nitrogen and nitrate levels, and the results indicated that both parameters were slightly lower in zone 1 than zone 2, although they did not differ significantly. Total contents of each metal; Cd, Co, Zn and Pb in the soil samples of zone 1 were significantly higher than those from zone 2 soils.

The available Cu concentration for plants in zone 1 consisted of 39% of total Cu level. There were no significant differences in the available levels of the studied metals between soils of the zones except for Cu. The soils of zone 2 did not display higher concentrations of other evaluated metals, among which Co and Cd were very low (Tab. 1). Accordingly, their levels could not be toxic for plants.

Measurement of Cu contents in roots, leaves and stems of *Alhagi camelorum* collected from the different sites showed that all tissues of our studied plant species in zone 1 contained significantly higher Cu levels as compared with samples collected from zone 2 soils. In addition, analysis showed significantly high Cu contents in leaf, stem and roots, in which, the level of Cu in leaves of *A. camelorum* rose up to around three fold of its total content in roots. The ratio of Cu in roots of this species growing on zone 1 to that in the roots of the same plants in zone 2 was approximately 7. Distribution of Cu in leaf

Tab. 1. Chemical characteristics of soils of the studied zones. Data were presented as mean ± SD. * – Significant difference with respect to zone 2 ($P < 0.05$). T – total content; E – DTPA – extractable content; EC – electric conductivity, # – electrolytic conductivity in water: soil extract (1:1).

Zone	Heavy metal content [mg (Kg dw)$^{-1}$]										pH	EC# (mS cm^{-1})	Nitrogen (g (kg dw^{-1})	
	Cu		Zn		Co		Cd		Pb					
	T	E	T	E	T	E	T	E	T	E			NO_3^-	Total
1	842.7 ±* 38.5	332.4 ±* 15.9	38.64 ±* 7.4	4.08 ± 0.36	9.6 ± 2.34	<0.11	3.05 ± 0.81	<0.17	12.8 ±* 3.61	<0.28	5.82 ± 0.44	2.37 ± 0.24	0.27 ± 0.03	1.41 ± 0.10
2	16.36 ± 3.52	4.21 ± 0.77	26.41 ± 4.9	6.24 ± 0.61	3.140 ± 1.83	<0.15	1.68 ± 0.56	<0.12	6.9 ± 1.82	<0.28	6.65 ± 0.25	1.92 ± 0.16	0.31 ± 0.02	1.68 ± 0.14

organelles revealed that the plant species in zone 1 had significantly higher Cu content in their vacuoles and/or chloroplasts than the same plant species in zone 2. The concentration of copper in vacuoles of *Alhagi camelthorn* was 29.67; this comprised 48% total leaf Cu levels and was over six times higher than the chloroplast Cu content (Tab. 2). The assay of biomass and chlorophyll contents showed that these parameters in plant species from zone 1 were insignificantly lower than in the same plant species associated with zone 2 (Tab. 3).

Tab. 2. Copper bioconcentration in tissues [g (kg dw)$^{-1}$] and in organelle [(μg (g dw^{-1})] of leaves. Data are presented as mean ± SD; ^{b} – Statistically different with respect to roots; ^{c} – significant difference as compared with vacuoles; statistically different with respect to stems; $^{\#}$ – The ratio of leaf organelle Cu to total leaf Cu

Zone	Tissue			Organelle			
	Leaf	Stem	Root	Chloroplasts	Cu/Cu%	Vacuoles	Cu/Cu%
1	61.54 ± 5.68 ^{b}	29.86 ± 3.24 ^{b}	17.05 ± 0.22	4.27 ± 0.71 ^{c}	7	29.67 ± 2.88	48
2	5.61 ± 1.05 ^{b}	3.28 ± 0.65	2.54 ± 0.49	0.28 ± 0.02 ^{c}	5	1.25 ± 0.18	22

Tab. 3. Chlorophyll content, and biomass of different plant tissues. Data are presented as mean ± SD.

Zone	Biomass mg (g FW)$^{-1}$		Chlorophyll mg (g FW)$^{-1}$
	Shoot	Root	
1	98.17 ± 5.64	76.41 ± 4.32	2.18 ± 0.24
2	112 ± 6.82	83.52 ± 5.16	2.71 ± 0.32

Tab. 4. Antioxidant enzyme activities and biomarkers of lipid peroxidation and protein oxidation in tissues of studied plants. Data are presented as mean ± SD. * – significant difference as compared with zone 2; $^{\#}$-significant difference as compared with roots**.**

Tissue	Zone	SOD (U/mg protein)	GPX (U/mg protein)	CAT (uM/min/mg)	MDA (nmol/mg protein)	Dityrosine (nmol/mg protein)	GSH (nmol/gFW)
leaf	1	28.10 ± 2.91 *#	5.41 ± 1.30 #	57.43 ± 5.32 *#	52.10 ± 4.12	4.36 ± 0.53	416 ± 26 *
	2	17.28 ± 2.62 #	6.26 ± 1.12 #	33.51 ± 2.81	23.62 ± 2.30	2.71 ± 0.22	670 ± 41
stem	1	31.58 ± 3.04 *#	8.10 ± 1.63 *	66.04 ± 5.84 *	45.70 ± 3.66	3.92 ± 0.26	285 ± 22 *
	2	12.77 ± 2.55	4.31 ± 0.82	40.55 ± 3.61	40.15 ± 3.85	3.21 ± 0.28	512 ± 38
root	1	38.26 ± 3.16 *	11.07 ± 1.74 *	87.12 ± 6.47 *	42.80 ± 3.30	3.10 ± 0.21	110 ± 14 *
	2	9.62 ± 1.64	3.72 ± 0.63	23.38 ± 2.31	47.32 ± 3.72	3.81 ± 0.35	367 ± 27

As a comparison, the antioxidant enzyme activities in tissues of *Alhagi camelorum* from zone 1, were significantly higher than those of zone 2. On the other hand, the increases in the enzyme activities of this species growing on zone 1 were considerable for roots, stems and then leaves, in that order, but in zone 2, the pattern of increase was observed in leaves, stems and then roots. Moreover, *A. camelorum* showed significant increase in SOD and CAT activities of roots as compared stems and/ or leaves in zone 1. GPX activity of the plant increased significantly only in the roots. With regard to the levels of oxidative damage biomarkers of lipids as MDA and proteins as dityrosine, these parameters were considerable in leaves, stems and roots in order in zone 1, but in zone 2 the pattern of increase was for roots, stems and leaves in that order. The studied parameters in the leaves of this plant species growing in zone 1 were significantly higher (about two fold) in comparison with the same plants collected from zone 2 (Tab. 4). In addition, the evaluated plant tissues from zone 1 revealed significantly lower concentrations of GSH than those from zone 2. By contrast, the levels of both PC_2 and PC_3 in each evaluated tissues of the studied plant from zone 1 were significantly higher than the same plant tissues collected from zone 2 (Fig 1).

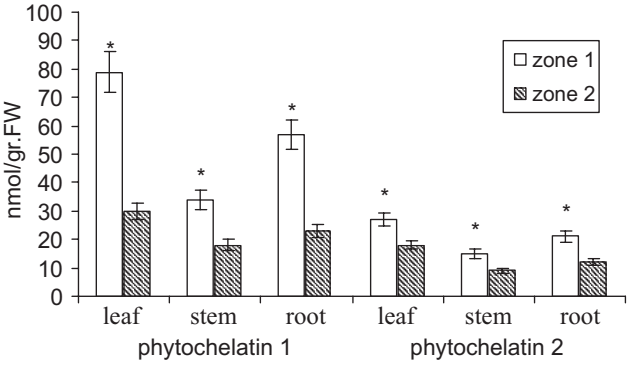

Fig. 1. The levels of phytochelatins in tissues of studied plants. * – significant difference as compared with zone 2.

Discussion

This investigation definitely showed that the study area was significantly contaminated with Cu at toxic levels and the rate of available concentration of this metal was high (about 39%) for plant growth. The copper levels in leaves of *A. camelorum* were above the critical level for copper toxicity (Robson and Reuter 1981). The soil analysis revealed normal levels of other heavy metals (Pb, Co and Cd). The high Cu availability may be attributed to the soil pH characteristic. Studies have confirmed that a low level of pH causes an increase in Cu solubility and its release from the soil phase leading to an elevation in copper uptake by roots (Watmough and Dickinson 1995). Our results were in accordance with the findings reported in the Cyprus Skouriotissa Cu mine, where pH was mildly acidic and contained copper up to 787 mg [kg (dry weight)]$^{-1}$ (Johansson et al. 2005). In our zones of investigation, the normal growth of our studied plant samples in metalliferous soils without any visual and conspicuous symptoms of Cu toxicity (Lewis et al. 2001) implied that *A. camelorum* was tolerant to toxic levels of Cu.

This plant species was endemic around the Cu mine and had adapted to contaminated soils by developing tolerance mechanisms to this metal stress. Most of these mechanisms have been recognized previously as exclusion, accumulation of metals and internal protective responses that vary among plant species and among different tissues affected by their intrinsic characteristics (FREITAS et al. 2004, NICOLAU et al. 2005, SHU et al. 2002, BRUN et al. 2001). *Alhagi camelorum* had the ability to accumulate copper in its tissues at levels that were lower than 1000 $\mu g\ g^{-1}$ tissue, a threshold limit that is prescribed for hyperaccumulators (REEVES and BAKER 2000).

Accordingly, our studied plant could not be considered as hyperaccumulator. With regard to the importance of Cu distribution, the patterns of copper bioaccumulation and partitioning among different parts of tolerant plants including *Paspalum distichum* and *Cynodon dactylon*, have been reported in many studies (MARSCHNER 1995, MULLIGAN et al. 2001, PYATT 2001, STOLTZ and GREGER 2002). In these two species that were collected from a Cu mine in China, the authors reported higher copper contents in roots of *Cynodon dactylon* and in the shoots of *Paspalum distichum* than in their other tissues. As another indication, *Pistacia terebinthus* and *Cistus creticus* collected from the Skouriotissa Cu mine accumulated a considerable amount of the absorbed Cu in their roots, although *Bosea cypria* accumulated most Cu in the leaves (JOHANSSON et al. 2005). In agreement with these documents, the studied plant, *A. camelorum,* showed Cu accumulation and partitioning in which Cu was transported and stored into the above ground parts, particularly into the leaves. The immobilization of excess heavy metals via their storage in cell walls (HUGHES and WILLIAMS 1988) or accumulation in vacuoles (MCCAIN and MARKLEY 1989), were suggested as another strategy to increase plant internal tolerance to Cu toxicity.

We found that *A. camelorum* from zone 1 accumulated about 48% of total Cu in the leaves vacuoles that was considerable as compared with the same plant collected from zone 2. Many studies have confirmed that the main portion of metals including copper enter the vacuoles in the form of a phytochelatin complex, where they gain acid-labile sulphur and form high molecular weight complexes (SANITA DI TOPPI et al. 2002). Potentially toxic heavy metal ions are firstly chelated by GSH and then transferred to PCs for eventual sequestration. However, GSH act as a first line of defense against metal toxicity by complexing metals before the induced synthesis of PCs arrives at effective levels (FREEDMAN et al. 1989, SINGHAL et al. 1987). PC synthesis induced by metals is accompanied by a rapid depletion of total GSH in plants, because GSH is the direct precursor/substrate for the synthesis of PCs (GUPTA et al. 1998). In conformity with these documents, we observed an elevation in PC levels and decrease in GSH concentrations in plants of zone 1 as compared to plants of zone 2. On the other hand and in agreement with our study, SUDHAKAR et al. (2006), showed that exposure of *Hydrilla verticillata* (L.f.) Royle to high doses of copper led to decrease in GSH and an elevation in PC levels. In addition, PCs usually play only a part in integrated mechanisms of copper homeostasis and detoxification. Tolerance strategies of plants to copper exposure may include other responses such as variation in antioxidative enzyme activities.

It has also been confirmed in many investigations that when copper is in excess, it can promote and stimulate generation of Fenton-type reactive oxygen species leading to increase in MDA and dityrosine as biomarkers of oxidative damages (WECKX and CLIJESTERS 1996, DEVI and PRASDA 1998, LOMBARDI and SEBASTIANI 2005). In response to ROS gener-

ation, antioxidant enzyme activities are elevated as defense system. These responses to excess copper can vary among plant species and among different tissues (LOMBARDI and SEBASTINI 2005). In this study, Cu-accumulation by vacuoles caused a decrease in the level of copper outside this organelle, leading to decrease in ROS production and low induction on antioxidative enzymes in the leaves as compared with roots of this plant.

Accordingly, the limited but significant increase in MDA and dityrosine in leaves as compared to the roots of *A. camelorum* could be the consequence of considerably low activities of antioxidative enzymes in leaves. However, the slight loss in chlorophyll content may be due to lipid peroxidation of chloroplast and thylakoid membranes and chlorophyll degradation mediated by Cu (BASZYNSKI et al. 1988, VINIT-DUNAND et al. 2002). In agreement with our findings, CHETTRI et al. (1998), reported a considerable decrease in total chlorophyll levels in *Cladonia convoluta* collected from a copper mine. They found a copper content in this plant species exceeding 175 $\mu g. g^{-1}$ dry weight. Moreover, although *A. camelorum* leaves in zone 1 showed a significant increase in oxidative damage parameters with respect to zone 2, the parameter of biomass decreased insignificantly in this tissue of the plant in zone 1. This could be attributed to insufficient activities of antioxidant enzymes within tissues of *A. camelorum* to protect them completely against ROS damage. However, the non-enzymatic antioxidant defense system comprises phytochelatins and GSH, functioning in free radical entrapment and metal detoxification, and restricted both the severe increase in MDA and dityrosine and a considerable decrease in the biomass parameter.

In conclusion, the present study revealed the involvement of the detoxification potential of phytochelatins, GSH, antioxidant enzyme activities and the ability to accumulate Cu in the leaf vacuoles in *A. camelorum* and protection against Cu-toxicity.

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References

AEBY, H., 1984: Catalase in vitro. Methods in Enzymology 105, 121–126.

AGATA, F., ERNEST, B., 1998: Metal–metal interactions in accumulation of V^{5+}, Ni^{2+}, Mo^{6+}, Mn^{2+} and Cu^{2+} in under and above ground parts of *Sinapis alba*. Chemosphere 36, 1305–1317.

ALAN, R. W., 1994: The spectral determination of chlorophyll a and b, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. Plant Physiology 144, 307–313.

ALSCHER, R. G., DONAHUE, J. L., CRAMER, C. L., 1997: Reactive oxygen species and antioxidants: relationships in green cells. Physiologia Plantarum 100, 224–233.

AMADO, R., AESCHBACH, R., NEUKOM, H., 1984: Dityrosine: in vitro production and characterization. Methods in Enzymology 107, 377–388.

BAKER, A. J. M., PROCTOR, J., 1990: The influence of cadmium, copper, lead and zinc on the distribution and evolution of metallophytes in British Island. Plant Systematics and Evolution 173, 91–108.

BASZYNSKI, T., TUKENDROF, M., RUSZKOWSKA, M., 1988: Characteristics of the photosynthetic apparatus of copper non-tolerant spinach exposed to excess copper. Journal of Plant Physiology 132, 708–713.

DUCHAUFOUR, P., 1970: Précis de Pedologie. Masson y Cie, Paris.

BIRD, B. R., HUNG, S. S. O., HADLEY, M., DRAPER, H. H., 1983: Determination of malonaldehyde in biological materials by high-pressure liquid chromatography. Analytical Biochemistry 128, 240–244.

BRADFORD, M. M., 1976: A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Analytical Biochemistry 72, 248–254.

BRUN, L. A., MAILLET, J., HINSINGER, P., PEPIN, M., 2001: Evaluation of copper-contaminated vineyard soils. Environmental Pollution 111, 293–302.

CHETTRI, M., COOK, C. M., VARADAKA, E., SAWIDIS, T., 1998: The effect of Cu, Zn and Pb on the chlorophyll content of the lichens *Cladonia convoluta* and *Cladonua rangformis*. Environmental and Experimental Botany 39, 1–10.

CLEMENS, S., 2001: Molecular mechanisms of plant metal tolerance and homeostasis. Planta 212, 475–486.

DEMIREVSKA-KEPOVA, K., SIMOVA-STOILOVA, L., STOYANOVA, Z., HOLZER, R., FELLER, U., 2004: Biochemical changes in barely plants after excessive supply of copper and manganese. Environmental and Experimental Botany 52, 253–266.

DEVI, S. R., PRASD, M. N. V., 1998: Copper toxicity in *Ceratophyllum demersum* L. a free floating macrophyte. Plant Science 138, 157–165.

DE VOS, C. H. R., VONK, M. J., VOOIJS, R., SCHAT, H., 1992: Glutathione depletion due to copper-induced phytochelatin synthesis causes oxidative stress in *Silene cucubalus*. Plant Physiology 98, 853–858.

DUCHAUFOUR, PH., 1970: Precis de Pedologie. Masson y Cie, Paris.

FREEDMAN, J. H., CIRIOLO, M. R., PEISACH, J., 1989. The role of glutathione in copper metabolism and toxicity. Journal of Biological Chemistry 264, 5598–5605.

FREITAS, H., PRASAD, M. N. V., PRATAS, J., 2004: Plant community tolerance to trace elements growing on the degraded soils of Sao Domingos mine in the south east of Portugal. Environment International 30, 65–72.

GUPTA, M., TRIPATHI, R. D., RAI, UN., CHANDRA, P., 1998: Role of glutathione and phytochelatin in *Hydrilla verticillta* (l. f.) Royle and *Vallisneria spiralis* under mercury stress. Chemosphere 37, 785–800.

HALLIWELL, B., GUTTERIDGE, J. M. C., 1998: Mechanisms of damage to cellular targets by oxidative stress: lipid peroxidation. In: HALLIWELL, B., GUTTERIDGE, J. M. C. (eds.), Free radicals in biology and medicine, 284–306. Oxford Science Publication, Oxford.

HEGEDUS, A., ERDEI, S., HORVATH, G., 2001: Comparative studies of H_2O_2 detoxifying enzymes in green and greening barley seedlings under cadmium stress. Plant Science 160, 1085–1093.

HERNANDEZ-JIMENZ, M. J., LUCAS, M. M., ROSARIO, M. F., 2002: Antioxidant defense and damage in senescing lupin nodules. Plant Physiology and Biochemistry 40, 645 – 657.

HOPKINS, J., TUDHOPE, G. R., 1973: Glutathione peroxidase in human red cells in health and disease. British Journal of Haematology 25, 563–575.

HUGHES, N. P., WILLIAMS, R. J. P., 1988: An introduction to manganese biological chemistry. In: GRAHAM, R. D., HANNAM, R. J., UREN, N. C. (eds.), Manganese in soils and plant, 7–19. Kluwer Academic, Dordrecht.

ICRCL, 1987: Inter-departmental committee on the redevelopment of contaminated land: Guidance notes (59/83). Guidance on the assessment and redevelopment of contaminated land. HMSO, London.

JOHANSSON, L., XYDAS, C., MESSIOS, N., STOLTS, E., GREGER, M., 2005: Growth and Cu accumulation by plants grown on Cu containing mine tailing in Cyprus. Applied Geochemistry 20, 101–107.

KABATA-PENDIAS, A., 1995: Agricultural problems related to excessive trace metals contents of soil. In: SALMONS, W., FORSTHER, U. (eds.), Concerning heavy metals: problems and solutions/salmons, 19–31. Springer-Verlag, Berlin.

KANTOL, M., SARRANEN, M., VANHA, P. T., 1998: Selenium and glutathione peroxidase in serum, plasma of men and bulls. Journal of Reproduction Fertility 83, 785–794.

KEENEY, D., NELSON, D., 1982: Nitrogen-inorganic forms. In: PAGE, A.L. (ed.), Methods of soil analysis, 2. ASA and SSSA, Madison, WI.

LEWIS, S., DONKIN, M. E., DEPLED GE, M. H., 2001: Hsp70 expression in *Enteromorpha intestinalis* (Chlorophyta) exposed to environmental stressors. Aquatic. Toxicology 51, 277-291.

LOMBARDI, L., SEBASTIANI, L., 2005: Copper toxicity in *Prunus cerasifera*: growth and antioxidant enzymes responses of in vitro grown plants. Plant Science 168, 797–802.

MARSCHNER, H. (Ed.), 1995: Mineral nutrition of higher plants. Academic Press, London.

MCCAIN, D. C., MARKLEY J. L., 1989: More manganese accumulates in maple sun leaves than in shade leaves. Plant Physiology 90, 1414–1421.

MCCORD, J., FRIDOVICH, I., 1969: Superoxide dismutase. An enzymatic function for erythrocuprein (hemocuprein). Journal Biology Chemistry 244, 6049–6055.

MEISTER, A., ANDERSON, M. E., 1983: Glutathione. Annual Review of Biochemistry 52, 711–760.

MINAMI, M., YOSHIKAWA, H., 1979: A simplified assay method of superoxide dismutase activity for clinical use. Clinica Chimica Acta 92, 337–342.

MULLIGAN, C. N., YONG, R. N., GIBBS, B. F., 2001: Remediation technologies for metal--contaminated soils and ground water: an evaluation. Engineering Geology 60, 193–207.

MORELLI, E., SCARANO, G., 2004: Copper-induced changes of non-protein thiols and antioxidant enzymes in the marine microalga *Phaeodactylum tricornutum*. Plant Science 167, 289–296.

NICOLAU, A., MARTINS, M. J., MOTA, M., LIMA, N., 2005: Effect of copper in the protistan community of activated sludge. Chemosphere 58, 605–614.

OGAWA, K., IWABUCHI, M., 2001: A mechanism for promoting the germination of *Zinnia elegans* seeds by hydrogen peroxide. Plant and Cell Physiology 42, 286–291.

ORHANL, H., VERMEULEN, N. P. E., TUMP, C., ZAPPEY, H., MEERMAN, J. H. N., 2004: Simultaneous determination of tyrosine, phenylalanine and deoxyguanosine oxidation products by liquid chromatography–tandem mass spectrometry as non-invasive biomarkers for oxidative damage. Journal of Chromatography B 799, 245–254.

PAGE, A. L., MILER, R. H., KEENEY, D. R., 1982: Methods of soil analysis, 2. Chemical and microbiological properties. American Society of Agronomy and Soil Science Society of America, Madison, Wisconsin.

PYATT, F. B., 2001: Copper and lead bioaccumulation by *Acacia retinoides* and *Eucalyptus torquata* in sites contaminated as a consequence of extensive Ancient mining activities in Cyprus. Ecotoxicology and Environmental Safety 50, 60–64.

REEVES, R., BAKER, A., 2000: Metal accumulating plants. In: RASKIN, I., ENSLEY, B. (eds.), Phytoremediation of toxic metals-using plants to clean up the environment, 193–229. John Wiley and Sons, New York,

ROBSON, A. D., REUTER, D. J., 1981: Diagnosis of copper deficiency and toxicity. In: LONERGAN, J. F., ROBSON, A. D., GRAHAM, R. D. (eds.), Copper in soils and plants, 287–312. Academic Press, London.

RUSINA, Y., KALOYAN, N., CHRISTOV, L., PETROVA, P., 2004: Antioxidative enzymes in barley plants subjected to soil flooding. Environment and Experimental Botany 51, 93–101.

SANITA DI TOPPI, L., PRASAD, M. N. V., OTTONELLO, S., 2002: Metal chelating peptides and proteins in plants. In: PRASAD, M. N. V., STRZAKO, K. (eds.), Physiology and biochemistry of metal toxicity and tolerance in plants, 59–93. Kluwer Academic.

SCHAT, H., LIUGANY, M.,VOOIJIS, R., HARTLEY-WHITAKER, J., BLEEKER, P. M., 2002: The role of phytochelatins in constitutive and adaptive heavy metal tolerances in hyperaccumulator and non-hyperaccumulator metallophytes. Journal of Experimental Botany 53, 2381–2392.

SCHOLZ-STARKE, J., ANGELI, A. D., FERRARETTO, C., PALUZZI, S., 2004: Redox-dependent modulation of the carrot SV channel by cytosolic pH. FEBS Letter 576, 449–454.

SHU, W. S., YE, Z. H., LAU, C. Y., ZHANG, Z. Q., 2002: Lead, zinc and copper accumulation and tolerance in populations of *Paspalum disticum* and *Cynodon dactylon*. Environmental Pollution 120, 445–453.

SIEB, C., 1995: Heavy metal availability to plants and soils irrigated with wastewater from Mexico City. Water Science Technology 32, 29–34.

SINGHAL, R. K., ANDERSON, M. E., MEISTER, A., 1987: Glutathion, a first line defense against cadmium toxicity. FASEB Journal 1, 220–223.

SNELLER, F. E. C., VAN HEERWAARDEN, L. M., KOEVOETS, P. L. M., VOOIJS, R., SCHAT, H., VERKLEIJ, J. A. C., 2000: Derivatization of phytochelatins from *Silene vulgaris*, induced upon exposure to arsenate and cadmium: comparison of derivatization with Ellman's reagent and monobromobimane. Journal of Agriculture and Food Chemistry 48, 4014–4019.

STADTMAN, E. R., OLIVER, C. N., 1991: Metal-catalyzed oxidation of proteins. The Journal of Biological Chemistry 266, 2005–2008.

STOLTZ, E., GREGER, M., 2002: Accumulation properties of As, Cd, Cu, Pb and Zn by four wetland plant species growing on submerged mine tailings. Environmental and Experimental Botany 47, 271–280.

SUDHAKAR, S., SEEMA, M., RUDRA, D. T., SANJAY, D., DHARMENDRA, K. G., 2006: Copper-induced oxidative stress and responses of antioxidants and phytochelatins in *Hydrilla verticillata* (L.f.) Royle. Aquatic Toxicology 80, 405–415.

VAN-ASSCHE, F., CLIJSTER, H., 1990: Effect of the metals on enzyme activity in plants. Plant Cell and Environment 13, 195–206.

VINIT-DUNAND, F., EPRON, D., ALOUI-LAOUI-SOSSE, B., BADOT, P. M., 2002: Effects of copper on growth and on photosynthesis of mature and expanding leaves in cucumber plants. Plant Science 163, 53–58.

WALDERMAR, M., RYSZARD, R., TERESA, U., 1994: Effect of excess Cu on the photosynthetic apparatus of runner bean leaves treated at two different growth stages. Physiology Plantarium 91, 715–721.

WATMOUGH, S. A., DICHINSON, N. M., 1995: Dispersal and mobility of heavy metals in relation to three survivals in an aerially contaminated woodland ecosystem. Environmental Pollution 90, 139–142.

WECHX, J. E. J., CLIJESTERS, H. M. M., 1996: Oxidative damage and defense mechanisms in primary leaves of *Phaseolus vulgaris* as a result of root assimilation of toxic amount of copper. Physiology Plantarium 96, 506–512.

YUAN, D. W., 1988: Compared study on the pre-treatment methods for measuring soil total copper, zinc, lead, cadmium, nickel and manganese. Agro-Environmental Protection Science 7, 34–36.